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EphrinB Ligands Recruit GRIP Family PDZ Adaptor Proteins into Raft Membrane Microdomains

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Summary

Transmembrane ephrinB proteins have important functions during embryonic patterning as ligands for Eph receptor tyrosine kinases and presumably as signal-transducing receptor-like molecules. Consistent with "reverse" signaling, ephrinB1 is localized in sphingo-lipid/cholesterol-enriched raft microdomains, platforms for the localized concentration and activation of signaling molecules. Glutamate receptor–interacting protein (GRIP) and a highly related protein, which we have termed GRIP2, are recruited into these rafts through association with the C-terminal PDZ target site of ephrinB1. Stimulation of ephrinB1 with soluble EphB2 receptor ectodomain causes the formation of large raft patches that also contain GRIP proteins. Moreover, a GRIP-associated serine/threonine kinase activity is recruited into ephrinB1–GRIP complexes. Our findings suggest that GRIP proteins provide a scaffold for the assembly of a multiprotein signaling complex downstream of ephrinB ligands.

Introduction

Eph receptor tyrosine kinases and their ephrin ligands play important roles in a variety of processes during embryonic development of mouse, chick, frog, fish, and worm. They mediate axon guidance and fasciculation, control migration of neural crest cells, and have important roles in patterning of embryonic structures (Orioli and Klein, 1997; Durbin et al., 1998; Flanagan and Vanderhaeghen, 1998; Yancopoulos et al., 1998). The ephrin–Eph system functions in cell–to–cell signaling, since Eph receptors are transmembrane proteins, and all ephrin ligands are tethered to the plasma membrane, either via a glycosylphosphatidylinositol (GPI) anchor (ephrinA ligands) or via a transmembrane region followed by an 83–90 amino acid cytoplasmic domain (ephrinB ligands) (Orioli and Klein, 1997). This subdivision matches the binding preferences of ephrinA and ephrinB molecules for the corresponding EphA or EphB receptor subfamilies, respectively, while within these subfamilies, interactions are highly promiscuous. With their highly conserved cytoplasmic domains, transmembrane ephrinB molecules resemble membrane receptors. Indeed, accumulating evidence points to an active signaling role for ephrinB molecules, resulting in "reverse" or—in combination with their ligand function—bidirectional signal transduction (Brückner and Klein, 1998; Holland et al., 1998).

It was proposed that kinase-independent functions for Eph receptors rely on the presence of the Eph extracellular domain serving as "ligand" to trigger ephrinB reverse signal transduction (Henkemeyer et al., 1996). Indeed, stimulation of ephrinB molecules by the EphB2 extracellular domain results in an ephrinB tyrosine phosphorylation response, representing the first and so far only biochemical evidence for ephrinB reverse signaling (Holland et al., 1996; Brückner et al., 1997). The mechanism of ephrinB tyrosine phosphorylation, however, has not been addressed so far. Furthermore, ephrinB ligands can participate in cross-talk with other signaling pathways as exemplified by ephrinB1, which undergoes tyrosine phosphorylation upon activation of platelet-derived growth factor receptor (PDGFR) (Brückner et al., 1997). The biochemical consequences of ephrinB tyrosine phosphorylation remain to be elucidated.

Localization is a major theme in signal transduction, as the recruitment of signaling molecules to the plasma membrane is a prerequisite for the functioning of various signaling cascades. Within the plasma membrane, correct localization is equally important, as recent work has shown that the membrane itself is highly organized into lipid domains that provide subcompartments. These small (<100 nm) membrane domains (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998), also called rafts, are enriched in sphingolipids and cholesterol and can incorporate GPI-anchored proteins, specific transmembrane proteins, and doubly acylated proteins like tyrosine kinases of the Src family (Simons and Ikonen, 1997). Rafts have been proposed to function as platforms for the assembly of cytoplasmic and membranous signaling molecules and for polarized membrane traffic (Holowka and Baird, 1996; Simons and Ikonen, 1997; Brown and London, 1998). The equilibrium of small raft domains can be changed by lateral clustering of membrane proteins, which then form large, microscopically visible patches of raft proteins and specific lipids, well segregated from nonraft proteins (Harder et al., 1998). Moreover, stabilized raft patches concentrate signaling molecules such as Src family tyrosine kinases, suggesting that these patches of raft domains serve as signaling centers (Harder et al., 1998). Several studies have suggested that the fusion of dispersed rafts to larger patches allows the interaction of intracellular signaling molecules and triggers signaling cascades (van den Berg et al., 1995; Stauffer and Meyer, 1997).

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Signal transduction requires a high degree of organization. In the signaling of membrane receptors, adaptor proteins provide docking sites for downstream enzymes in proximity to the activated receptor. A variety of adaptor proteins with modular SH2, SH3, or phosphotyrosine binding (PTB) protein–protein interaction domains have been described (Pawson and Scott, 1997). Recently, a number of proteins containing PDZ (PSD-95, Dlg, ZO-1) domains (Kornau et al., 1997; Craven and Bredt, 1998) have been recognized as having adaptor function. PDZ proteins were originally thought to mediate the concentration of neurotransmitter receptors and ion channels at the synapses of neurons, as well as the asymmetric distribution of receptors in epithelial cells or their localization at tight junctions (Sheng, 1996; Kim, 1997; Bredt, 1998). Some of these PDZ proteins have a dual function, serving also as scaffolds for the assembly of multiprotein signaling complexes at the membrane (Ranganathan and Ross, 1997; Tsunoda et al., 1997). PDZ domains represent modular domains of 80–100 amino acids that are found in a large variety of cytoplasmic proteins (Ponting et al., 1997). They mediate protein–protein interactions, generally recognizing short C-terminal motifs. Originally described as the T/SXV motif, the consensus sequence of PDZ binding sites meanwhile has been extended (Songyang et al., 1997). Interestingly, one such consensus PDZ binding motif, −YKV, is also present in the C terminus of all transmembrane ephrinB molecules (Bergemann et al., 1998).

Here, we describe the interaction of ephrinB ligands with the multi-PDZ domain protein glutamate receptor–interacting protein (GRIP) (Dong et al., 1997) and a novel, highly related protein, which we have termed GRIP2. A shorter isoform of GRIP2 was independently isolated as AMPA receptor–binding protein and termed ABP (Srivastava et al., 1998). Moreover, ephrinB1 is associated with sphingolipid/cholesterol-rich raft membrane microdomains and recruits a significant fraction of GRIP proteins from the cytoplasm into these rafts. Stimulation with EphB2 receptors reorganizes ephrinB1-containing rafts into larger raft patches that concentrate GRIP proteins on the cell surface. Furthermore, GRIP proteins are associated with a serine/threonine kinase activity when forming a complex with ephrinB ligands. In summary, we propose a novel mechanism for ephrinB-mediated signaling through its association with membrane microdomains and clustering into raft patches. We further suggest a novel function for GRIP proteins in the assembly of a multiprotein signaling complex downstream of ephrinB molecules.

Results

The Cytoplasmic Domain of EphrinB1 Binds GRIP1 and GRIP2, a Family of PDZ Adaptor Proteins

In an effort to study the mechanism of ephrinB function, we searched for proteins that would bind the cytoplasmic domain of ephrinB1. We used the entire cytoplasmic domain of human ephrinB1 as bait to screen a human fetal brain cDNA library in a yeast two-hybrid assay. Two independent isolates represented the same clone (5), which encoded a 5′ truncated version of a human cDNA highly similar to rat GRIP (Dong et al., 1997). Amino acid sequence identity of the human clone with rat GRIP was 94%, suggesting that it represented the human ortholog of GRIP. Clone 5 encoded a partial PDZ domain 3 (PDZ3) and an intact PDZ4 through PDZ7 (corresponding to amino acids 265–1112 of the published rat cDNA). The interaction with ephrinB1 was specific and was abolished by deletion of the last seven amino acids of ephrinB1 (data not shown). This indicated that GRIP bound a region containing the PDZ consensus motif (−YYXV*) at the ephrinB1 C terminus. Moreover, substitution of the last seven amino acids for the C terminus of the NMDA receptor subtype R2A (−SDV*), which specifically binds the PDZ domain proteins like PSD-95 (Kornau et al., 1995), did not restore binding to GRIP (data not shown).

In a PCR search for GRIP-related proteins, we isolated a novel GRIP-related cDNA, which we have termed GRIP2 (we will refer to the original GRIP clone as GRIP1). The screen of a rat hippocampal cDNA library yielded three independent cDNA clones of GRIP2. A 5 kb cDNA clone contained a 3006 bp open reading frame encoding a 1002 amino acid protein with a predicted molecular weight of 107.7 kDa. A second 3 kb cDNA clone contained an in-frame insertion of 123 bp, encoding an additional stretch of 41 amino acids in the first linker region between PDZ3 and PDZ4. The longest open reading frame therefore encoded a putative protein of 1043 amino acids. Recently, a partial rat cDNA clone of GRIP2 was published as AMPA receptor–binding protein (ABP) (Srivastava et al., 1998). Areas of overlaps between GRIP2 and ABP are indicated in Figure 1. GRIP2 contains an additional 52 amino acids at its N terminus that have 56% identity to GRIP1 in this region, indicating that this stretch is still part of the open reading frame. Moreover, in contrast to ABP, the GRIP2 cDNA exhibits the same domain structure as GRIP1, with seven PDZ domains and an overall 59% identity to rat GRIP1. We therefore consider these two proteins the founding members of a protein family that we will refer to as the GRIP family of adaptor proteins. The linker regions between PDZ3 and PDZ4, and between PDZ6 and PDZ7, are much less conserved between GRIP1 and GRIP2 (Figure 1B). These linker regions are rich in proline and serine residues, suggesting the possibility of specific interactions with other proteins or specific modifications such as phosphorylation (see below).

The region of GRIP1 required for the interaction with AMPA receptors comprised PDZ4, together with PDZ5 and 30 amino acids on the N-terminal side of PDZ4 (Dong et al., 1997). To map the essential GRIP domains for interaction with ephrinB1, deletion constructs of GRIP1 and GRIP2 were generated and tested for binding to the ephrinB1 cytoplasmic domain with the yeast two-hybrid system. As shown in Figure 2, only those deletion constructs of GRIP1 (G1) and GRIP2 (G2) that contained an intact PDZ6 demonstrated strong binding, including G1/4–6, G1/5+6, G1/6+7, and G1/6 and corresponding GRIP2 constructs. Constructs in which PDZ6 was partially deleted did not show specific binding, with the exceptions of G1/3p-5 and G2/7, which conferred weak binding, suggesting that other PDZ domains can have some affinity for the ephrinB1 C terminus as well. These results demonstrate that PDZ6 of GRIP1 and GRIP2 is sufficient to mediate binding to ephrinB1. Interestingly,

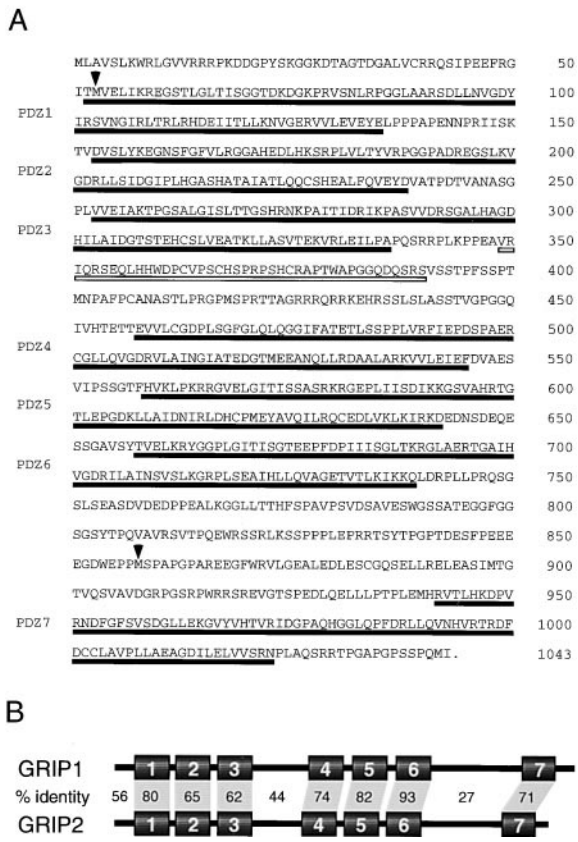

Figure 1. Structure of GRIP2

(A) Amino acid sequence of rat GRIP2. The depicted sequence contains a 41 amino acid insert in the linker region between PDZ3 and PDZ4 (indicated by an open horizontal bar). Individual PDZ domains are underlined by closed horizontal bars. N- and C-terminal amino acids of the shorter ABP (GRIP2) cDNA clone (Srivastava et al., 1998) are indicated by arrowheads.

(B) Comparison of rat GRIP1 and rat GRIP2. PDZ domains are indicated by boxes. Percent amino acid identities between PDZ domains and linker regions are shown.

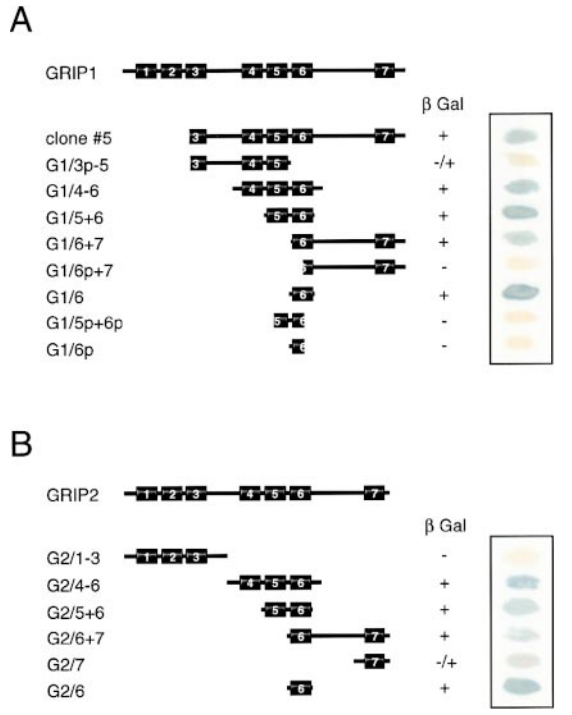

Figure 2. Mapping of PDZ Domains of GRIP1 and GRIP2 Required for Interaction with EphrinB1

Deletion mutants of GRIP1 (A) and GRIP2 (B) were used in the yeast two-hybrid assay to test for interaction with the cytoplasmic region of ephrinB1. β-Galactosidase activity was assayed by growth on X-gal-containing SD plates. Color reactions were judged to be strong (+), weak (−/+), or negative (−). Partial PDZ domains (p) are indicated where appropriate.

this region shows the highest sequence similarity between the two GRIP family members (Figure 1B). Both GRIP1 and GRIP2 therefore represent candidates for intracellular binding partners of ephrinB proteins.

Overlapping Expression Patterns of EphrinB Ligands and GRIP Family Proteins

Since ephrinB ligands have important roles during pattern formation in the developing embryo, we used in situ hybridization analysis to investigate ephrinB expression in comparison to GRIP1 and GRIP2 expression during embryogenesis to assess the degree of colocalization. As the transmembrane ephrinB ligands have conserved intracellular regions that all contain the C-terminal PDZ binding motif −YKV*, we included all three known ephrinB ligands (ephrins B1–B3) in our analysis.

Nervous System

In embryonic day 17 (E17) rat embryos, ephrinB1 and ephrinB2 showed overlapping expression, with highest levels in the forebrain, including cerebral cortex, striatum, and olfactory bulb, and in the inferior colliculus of the midbrain (Figure 3). EphrinB2 was expressed at low levels throughout the CNS, while ephrinB1 expression was absent in ventral forebrain, midbrain, and spinal cord. Both ephrinB1 and ephrinB2 were expressed in dorsal root ganglia. Interestingly, with the exception of the inferior colliculus and olfactory bulb, ephrinB3 expression was largely complementary to ephrinB1 and ephrinB2, with low levels in cerebral cortex and striatum and much higher expression in the ventral forebrain and the rest of the CNS. GRIP1 was expressed relatively homogeneously and strongly throughout most regions of the E17 embryonic rat nervous system. GRIP2 showed strong expression in the cortical plate of the cerebral cortex, olfactory bulb, and inferior colliculus. Based on coexpression throughout most of the CNS, GRIP1 and ephrinB3 have the greatest potential to interact. All ephrins and GRIPs were coexpressed at varying levels in the cortical plate of the cerebral cortex. All ephrins and GRIP1, but not GRIP2, were also expressed in ventricular and intermediate zones of the cerebral cortex. In addition, olfactory bulb and posterior midbrain strongly express ephrinB ligands and GRIPs. In the peripheral nervous system, dorsal root ganglia coexpressed GRIP1, ephrinB1, and ephrinB2. Earlier in development (E12 and E14), homogeneous GRIP1 expression was observed throughout the CNS, whereas GRIP2 expression levels were restricted to the spinal cord at E12 (Figure 3) and increased throughout the CNS at E14 (data not shown). The patterns of ephrinB expression

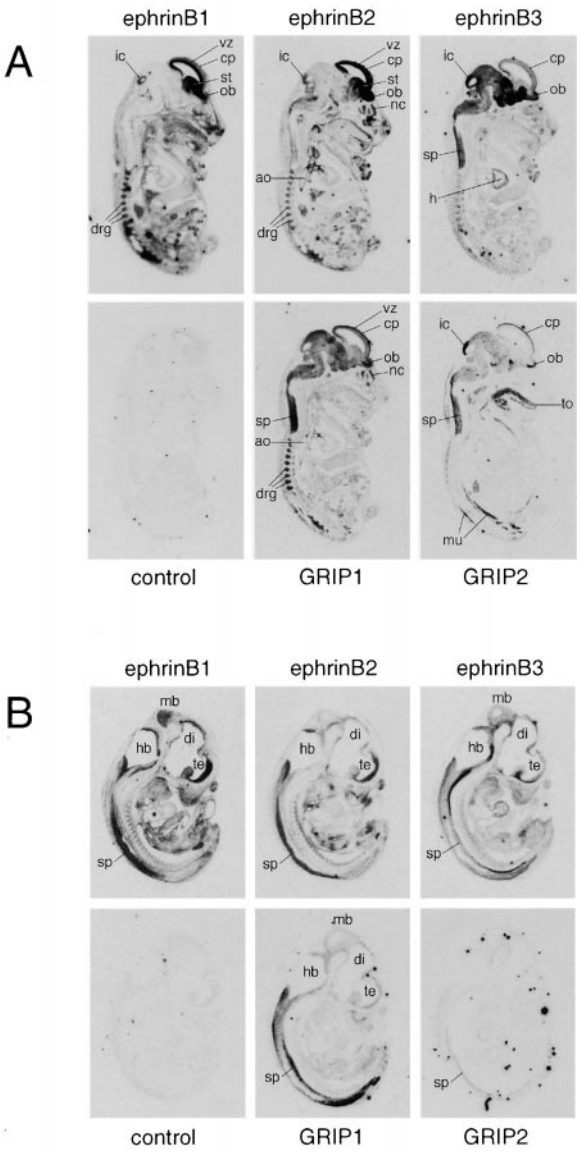

Figure 3. Colocalization of EphrinB Ligands and GRIPs in Mouse Embryos

In situ hybridization of E17 (A) and E12 (B) rat embryos using the indicated ^{35}S-labeled oligonucleotide probes. In control panels, 100-fold excess of unlabeled ephrinB1 oligo was used in the ephrinB1 in situ hybridization. All other control in situ hybridizations gave similar low background (data not shown). Abbreviations: ao, dorsal aorta; cp, cortical plate; di, diencephalon; drg, dorsal root ganglion; h, heart; hb, hindbrain; ic, inferior colliculus; mb, midbrain; mu, muscle; nc, nasal cavity; ob, olfactory bulb; sp, spinal cord; st, striatum; te, telencephalon; to, tongue; and vz, ventricular zone.

were established by E12 and remained relatively stable during the time period studied (E12–E17) (Figure 3 and data not shown). In postnatal brain, GRIP1 colocalized with ephrinB2 and ephrinB3 in hippocampus, neocortex, and olfactory bulb (data not shown).

Nonneuronal Tissues

As in the nervous system, expression of ephrinB1 and ephrinB2 was overlapping and seen in many tissues, whereas ephrinB3 was expressed at much lower levels, with the exception of the heart (Figure 3). GRIP1 was expressed at very low levels in many tissues; higher levels were found in nasal cavities and major blood vessels. GRIP2 expression outside the nervous system was mostly confined to muscle, including the tongue, where it partially colocalized with ephrinB1 and ephrinB2. In light of the essential role of ephrinB2 in blood vessel formation (Wang et al., 1998; Adams et al., 1999), it was interesting to note coexpression of GRIP1, ephrinB2, and ephrinB1 in aortic outflow tracts of the heart and dorsal aorta. Overlapping expression of ephrinB2 and GRIP1 was also seen in the nasal cavities. These results suggest roles for GRIP proteins during embryonic development in conjunction with ephrinB proteins.

EphrinB Ligands Associate with Both GRIP Proteins in Mammalian Cells and Mouse Brain

To evaluate the interaction of ephrinB1 and GRIPs in mammalian cells, we cotransfected HEK 293 cells with ephrinB1 carrying an N-terminal hemagglutinin- (HA-) epitope tag (HA–ephrinB1) together with N-terminally *myc*-tagged GRIP1 corresponding to the isolated partial clone 5. HA–ephrinB1 was expressed on the cell surface as verified by specific binding to soluble EphB2–alkaline phosphatase fusion protein (data not shown). As shown in Figure 4A, ephrinB1 specifically coimmunoprecipitated with *myc*–GRIP1 only from cells that had been cotransfected with *myc*–GRIP1. Moreover, this interaction could be partially affected by addition of a large excess (20 μg/ml) of a peptide comprising the last seven amino acids of ephrinB1, indicating that in HEK 293 cells, GRIP1 associated with the most C-terminal residues of ephrinB1. Consistent with this notion, a cell surface–expressed C-terminally truncated version of ephrinB1 lacking the cytoplasmic region (HA–ephrinB1ΔC) failed to bind *myc*–GRIP1 (Figure 4A).

The cytoplasmic region of the three known ephrinB ligands is highly conserved (Gale et al., 1996), although the cytoplasmic region of ephrinB3 is more divergent from both ephrinB1 and ephrinB2 (Bergemann et al., 1998). Whereas the 33 C-terminal amino acids of ephrinB1 are identical to ephrinB2, ephrinB3 has three amino acid substitutions, including a proline residue in place of alanine at position −7 from the C terminus. We therefore examined the interaction of ephrinB1 and ephrinB3 with both GRIP proteins in HEK 293 cells. Both ephrins and GRIP members were expressed at similar levels, respectively (Figure 4B). GRIP1 and GRIP2 coimmunoprecipitated with full-length ephrinB1 and ephrinB3, whereas C-terminally truncated versions of both ephrinB ligands failed to associate with the GRIP proteins. We consistently observed that significantly less *myc*–GRIP2 than *myc*–GRIP1 was immunoprecipitated with anti-*myc* antibodies, although the amounts of coprecipitated ephrinB ligands were similar for both GRIP members (Figure 4B). These results suggest that ephrinB ligands interact more stably with GRIP2 than with GRIP1. However, we cannot exclude the possiblity that ephrin association might render the pool of ephrin-bound GRIP2 protein particularly accessible for immunoprecipitation by the anti-*myc* antibody.

To investigate if ephrinB ligands associate with GRIP proteins in the mouse embryo, we immunoprecipitated endogenous ephrinB proteins from E14 mouse embryo heads and assayed for coimmunoprecipitated GRIP1 by

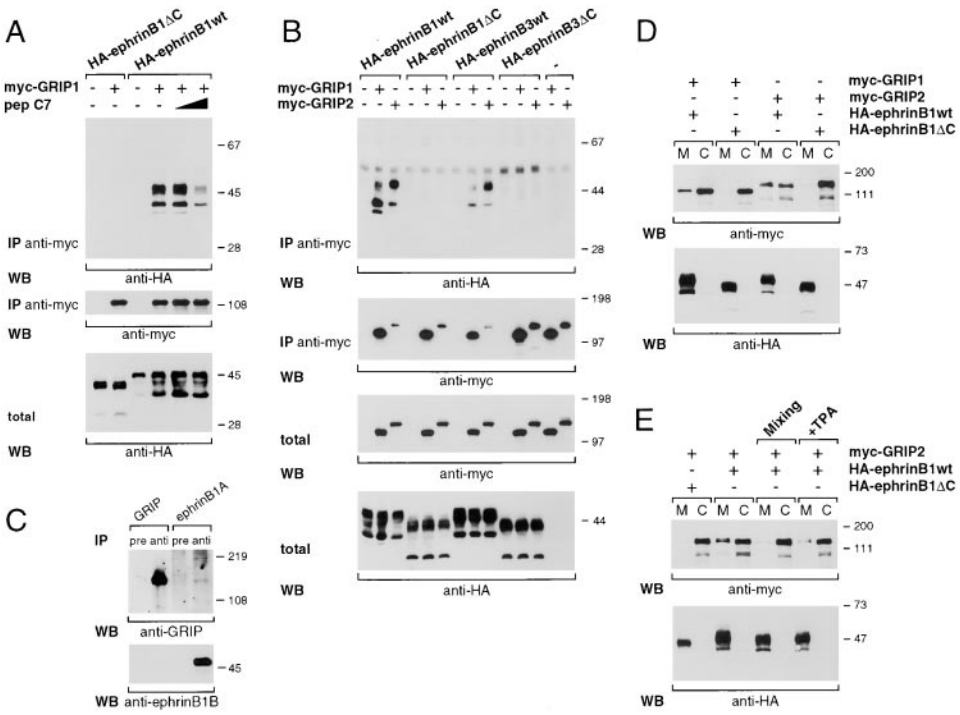

Figure 4. EphrinB Ligands Bind and Recruit GRIP Proteins to Cellular Membranes in HEK 293 Cells

(A) N-terminally HA-tagged ephrinB1 (HA–ephrinB1) associates with *myc*-tagged partial GRIP1 (PDZ3p-7) when coexpressed in HEK 293 cells. HEK 293 cells transiently expressing wild-type (wt) HA–ephrinB1 (HA–ephrinB1wt) or ephrinB1 lacking the cytoplasmic region (HA–ephrinB1ΔC), either alone or in combination with *myc*–GRIP1, were lysed and immunoprecipitated with anti-*myc* antibodies. IPs were analyzed by 10% SDS–PAGE and immunoblotted (WB) with anti-HA antibodies to visualize coimmunoprecipitated ephrinB1. Blots were stripped and reprobed with anti-*myc* antibodies to visualize GRIP1 protein levels. Aliquots of the same lysates were directly run on a separate SDS–PAGE and probed with anti-HA to visualize HA–ephrinB1 protein levels (bottom panel). Incubation of lysates with excess amounts of peptide (0.2 μg/ml; 20 μg/ml) corresponding to the seven C-terminal amino acids of ephrinB (pep C7) reduced the association between ephrinB1 and GRIP1 in this assay.

(B) Both GRIPs associate with ephrinB1 and ephrinB3 in HEK 293 cells. The assay was essentially done as in (A). HEK 293 transiently expressing HA–ephrinB1wt, HA–ephrinB1ΔC, HA–ephrinB3wt, or HA–ephrinB3ΔC, either alone or in combination with *myc*–GRIP1 or full-length *myc*–GRIP2, were assayed for ephrinB–GRIP interactions. To achieve satisfactory expression levels of ephrinB3 (bottom panel), a chimeric protein was used, consisting of the extracellular domain of ephrinB1 and transmembrane and cytoplasmic regions of ephrinB3 (see Experimental Procedures). Note that similar amounts of wild-type but not C-terminally truncated ephrinB1 or ephrinB3 coimmunoprecipitate with GRIP proteins. IPs using anti-*myc* antibodies consistently contained less GRIP2 than GRIP1 protein (second panel from top), suggesting that the *myc* tag was less accessible. Presumably as a result from the overexpression system, faster migrating ephrin bands are observed that may represent underglycosylated ephrin species.

(C) Coprecipitation of ephrinB1 and GRIP1 from embryo lysates. Protein lysates were prepared from E14 embryo heads, immunoprecipitated with either preimmune serum (Pre) or an antiserum to ephrinB1 (ephrinB1A), and immunoblotted with an antiserum to GRIP1. The stripped blot was reprobed with a second antiserum to ephrinB1 (anti-ephrinB1B). In the converse experiment, coimmunoprecipitation of ephrinB1 with GRIP could not be observed.

(D) EphrinB1 recruits GRIP1 and GRIP2 into membrane fractions. HEK 293 cells were transiently transfected with HA–ephrinB1wt, HA–ephrinB1ΔC in combination with *myc*-tagged GRIP1, or GRIP2. Cell homogenates were subjected to a flotation gradient membrane fractionation assay and were analyzed for the presence of GRIP and ephrinB1 proteins in membranous (M) and cytoplasmic (C) fractions. In the presence of wild-type ephrinB1, a large proportion of GRIP1 and GRIP2 are associated with membranes, while in the presence of ephrinB1ΔC, GRIP proteins are solely cytoplasmic.

(E) GRIP membrane localization is regulated. In vitro mixing of cell homogenates of separately expressed GRIP2 and ephrinB1wt does not result in GRIP2 membrane localization (Mixing), proving that ephrin–GRIP association occurs in the intact cell. Overnight treatment with TPA releases GRIP from ephrinB1-containing cellular membranes, demonstrating regulation of the ephrin–GRIP interaction.

immunoblotting. As shown in Figure 4C, in anti-ephrinB1 immunoprecipitates (IPs), but not in preimmune controls, a GRIP-immunoreactive protein is coprecipitated, albeit with low efficiency. This protein shows the same migration behavior in SDS-polyacrylamide gel electrophoresis (SDS–PAGE) as GRIP protein from control embryo lysate using anti-GRIP1 antiserum. In the converse experiment, presumably due to technical reasons, coimmunoprecipitation of ephrinB1 with GRIP protein could not be observed. In native tissue, ephrin–GRIP interactions may be more complex, due to the presence of additional ephrin- and GRIP-competing interaction partners. Thus, less efficient coimmunoprecipitations between GRIP and ephrin proteins would be expected. However, the experiment may be improved by the selective use of highly coexpressing tissue areas as a source for protein extracts.

EphrinB Ligands Recruit GRIP Proteins to Cellular Membranes

We next performed membrane fractionation assays to test whether interaction of GRIP proteins with ephrinB1

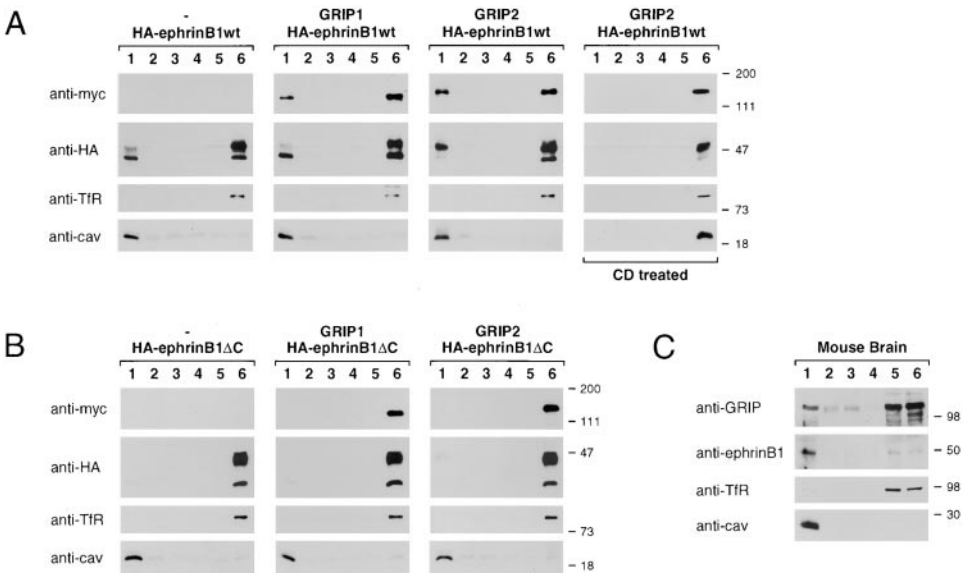

Figure 5. EphrinB1 Recruits GRIP Proteins into Raft Membrane Microdomains

HEK 293 cells transiently expressing HA–ephrinB1wt (A), HA–ephrinB1ΔC (B) either alone or together with *myc*-tagged GRIP1, or GRIP2 were subjected to TX100 extraction followed by Optiprep flotation gradients. Six fractions were collected from top to bottom and analyzed by Western blotting.

(A) The DIG-enriched fraction 1 contained HA–ephrinB1wt (anti-HA), *myc*–GRIP2 (anti-*myc*), and the raft protein caveolin (anti-cav) but not the nonraft membrane protein transferrin receptor (anti-TfR). Cyclodextrin treatment (CD) abolished the association of *myc*–GRIP2, HA–ephrinB1wt, and caveolin with membrane microdomains.

(B) Neither HA–ephrinB1ΔC (anti-HA) nor *myc*–GRIP2 (anti-*myc*) are detected in the DIG-enriched fraction 1, which does contain the control raft marker caveolin (anti-cav).

(C) A membrane preparation of an adult mouse brain homogenate was subjected to TX100 flotation. Six fractions from top to bottom were analyzed by Western blotting for the presence of GRIP and ephrinB proteins. The majority of ephrinB protein is found in the DIG-enriched fraction 1, indicating raft localization. Consistent with ephrin interaction, GRIP partially copurifies with fraction 1. Caveolin and transferrin receptor serve as raft-associated and nonraft control proteins, respectively, confirming specificity of the preparation.

would determine the subcellular localization of GRIP. Using a flotation gradient to separate membranes from cytoplasmic proteins, we observed that in cells coexpressing either GRIP1 or GRIP2 and the C-terminally truncated HA–ephrinB1ΔC, GRIP proteins were present in the cytoplasmic fraction at the bottom of the gradient. In contrast, in the presence of wild-type ephrinB1, a large portion of GRIP proteins was recruited to the floating membranous fraction, indicating that the interaction with ephrinB1 confers membrane association of both GRIP proteins (Figure 4D). This demonstrated that GRIP1 is recruited to ephrinB1-containing membranes similar to GRIP2, although coimmunoprecipitation experiments had suggested that ephrin–GRIP1 interactions may be less stable. Reduced stability of ephrin–GRIP1 interactions could be due to N-terminal deletions of the partial GRIP1 clone used. In vitro mixing of cell homogenates containing separately expressed ephrinB1wt and GRIP2 was not sufficient to achieve GRIP membrane association (Figure 4E). This indicated that biochemically observed ephrin–GRIP interactions indeed take place in the intact cell. As expected, wild-type and C-terminally truncated HA–ephrinB1ΔC were always recovered in the membranous fraction (Figures 4D and 4E). The amount of membranous GRIP varied between experiments and ranged from 20% to 90% of total GRIP protein. We therefore asked if ephrinB–GRIP interactions could be regulated by external signals. Indeed, we found that overnight treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), a general activator of cell proliferation and migration, consistently reduced the amount of membranous GRIP protein, possibly by regulating the phosphorylation state of either ephrinB or GRIP proteins. These results demonstrate the specific and regulated interaction of GRIP adaptor proteins with the cytoplasmic domain of ephrinB1 in plasma and/or intracellular membranes of mammalian cells.

EphrinB1 Recruits GRIP Proteins into Raft Membrane Microdomains

In an attempt to address the functional significance of ephrinB–GRIP interactions, we analyzed whether these complexes occurred in raft membrane microdomains, which have been implicated in processes such as sorting in polarized cells and signal transduction. Triton X-100- (TX100-) insoluble raft membrane proteins can be identified by their ability, due to their high lipid content, to float in TX100-containing density flotation gradients as detergent-insoluble glycolipid-enriched complexes (DIGs) (Brown and Rose, 1992). This property clearly distinguishes them from insoluble complexes formed by the cytoskeleton. As shown in Figure 5, different combinations of GRIP1 or GRIP2 and both wild-type and C-terminally truncated ephrinB1 were expressed in HEK 293 cells. While truncated ephrinB1ΔC, despite its plasma membrane localization (Figures 4D and 4E), is

not associated with DIGs, a large fraction of the full-length form (HA–ephrinB1wt) is found in the top fractions of the flotation gradient, indicating raft localization (Figure 5). Copurification of the raft protein caveolin in DIGs and full solubilization of nonraft membrane proteins such as the transferrin receptor confirm the quality of the preparation. This demonstrates that ephrinB1 localization in raft membrane microdomains relies on the presence of its cytoplasmic domain. When coexpressed with wild-type ephrinB1, but not ephrinB1ΔC, major proportions of GRIP proteins are detected in DIG fractions of the flotation gradient, indicating recruitment by and colocalization with ephrinB1 in raft microdomains (Figures 5A and 5B). As with the membrane fractionation experiments, we observed a certain variability in the proportion of raft-associated GRIP protein. Similar to TPA-induced GRIP release from the membrane, this points to a physiological regulation of the GRIP–ephrin interaction. To further assess the specificity of raft association of the ephrinB1–GRIP complexes, we used methyl-β cyclodextrin to deplete cholesterol from the cells. This treatment was previously shown to disrupt raft domains, resulting in increased TX100 solubility of their components (Scheiffele et al., 1997). Consistently, after incubating HEK 293 cells with 10 mM methyl-β cyclodextrin, no ephrinB1–GRIP2 complexes were found floating in the density gradient (Figure 5A). Next, we performed flotation gradients after TX100 extraction to assay for the presence of ephrinB in raft microdomains using lysates derived from membrane preparations of adult mouse brains. In Figure 5C, the majority of ephrinB proteins are found in the lipid-associated fraction 1, indicating localization in rafts. A sizable fraction of GRIP protein copurifies with ephrinB ligands, demonstrating localization in lipid microdomains and suggesting recruitment to these domains by association with ephrinB proteins. Localization of caveolin in the first and transferrin receptor in the last fractions of the flotation gradient demonstrates the specificity of the experiment. These results show that consistent with ephrin signaling, ephrinB1 is localized in raft membrane microdomains from transfected mammalian cells and primary mouse tissue. Furthermore, ephrinB1 is sufficient to recruit a significant proportion of GRIP proteins into lipid raft domains, giving GRIP proteins the chance to participate in ephrinB signaling.

Extracellular Clustering of EphrinB1 Recruits GRIP2 into Large Patches on the Cell Surface

We next wanted to observe the behavior of ephrinB1–GRIP complexes in living cells. Lateral cross-linking of membrane proteins has previously been shown to create stabilized raft patches that may represent signaling centers (Harder et al., 1998). Involvement of GRIP proteins in signaling by ephrinB1 would require a significant fraction of GRIP to be recruited into ephrinB1-containing patches. A green fluorescent protein (GFP) tag was attached to the extracellular N terminus of ephrinB1, and this construct was expressed in BHK cells, together with GRIP2. Fluorescence of GFP revealed a uniform staining of the plasma membrane (Figure 6). Similar surface staining was observed when unpermeabilized cells were labeled with anti-GFP antibodies (data not shown).

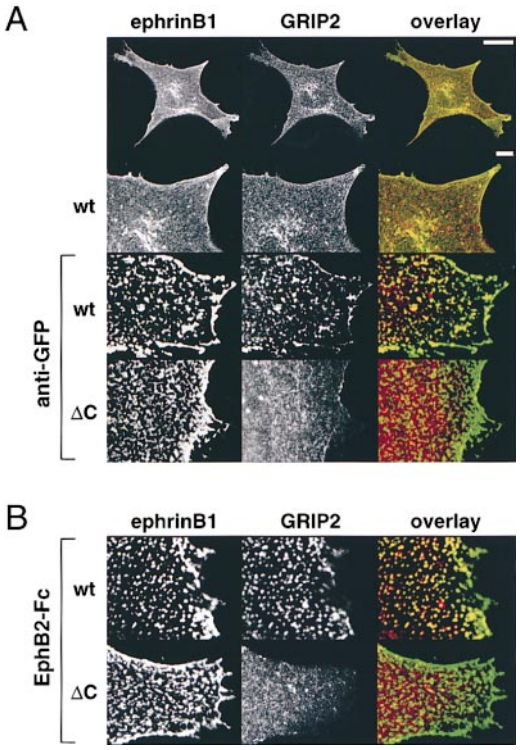

Figure 6. Copatching of EphrinB1 and GRIP2 after Crosslinking and EphB2 Receptor Binding

(A) BHK cells transiently expressing GFP–ephrinB1 containing either an intact cytoplasmic domain (wt) or a cytoplasmic truncation (ΔC), together with *myc*–GRIP2, were either left untreated or treated with anti-GFP antibodies to cross-link ephrinB1. The left column shows GFP fluorescence or anti-GFP signal, respectively, the middle column GRIP2 distribution, and the right column the merging of the two signals. Scale bars, 10 μm in top panel and 2.5 μm in second panel.

(B) BHK cells were transiently transfected with either wild-type (wt) ephrinB1, or C-terminally truncated ephrinB1ΔC and GRIP2. Cells were treated with EphB2–Fc receptor bodies recognizing ephrinB1. The left column shows bound EphB2 by anti-Fc immunofluorescence, the middle column GRIP2 distribution, and the right column the merge of the two signals. Note that in the presence of ephrinB1ΔC, GRIP2 is not redistributed into patches.

Some ephrinB1 was found associated with intracellular membranes most likely representing the Golgi complex. Epitope-tagged GRIP2 detected by anti-*myc* antibodies and immunofluorescence was found throughout the cell and colocalized with ephrinB1 on the intracellular membranes. Only in cells expressing very high levels of GRIP protein, we observed large intracellular aggregates that did not colocalize with ephrinB1 (data not shown). Spontaneous large clusters of ephrinB1, with GRIP2 at the cell surface, were observed in very few cases (data not shown). Anti-GFP antibodies were used to laterally cross-link ephrinB1 in living cells. This caused a redistribution of ephrinB1 into large patches on the cell surface. Interestingly, GRIP2 was also redistributed into patches that closely overlapped with ephrinB1 patches, showing tight association between these proteins (Figure 6). In contrast, extracellular cross-linking of C-terminally truncated GFP–ephrinB1ΔC did not cause the formation of GRIP2 copatches, clearly showing that the association

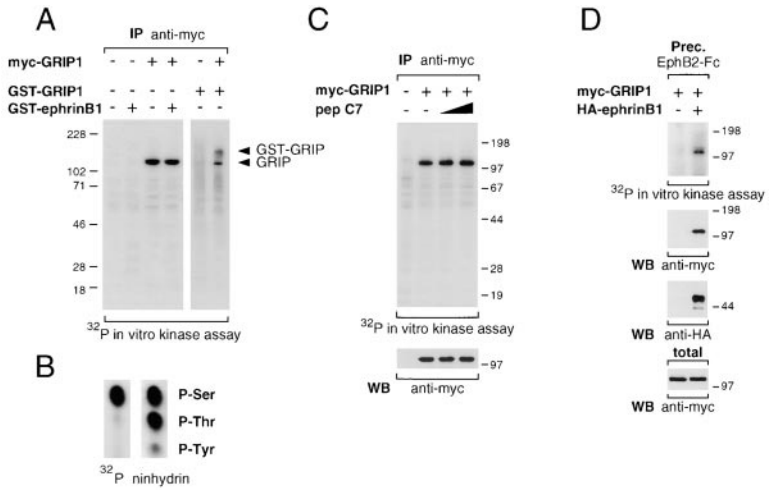

Figure 7. Recruitment of a Serine/Threonine Kinase to the Ephrin–GRIP Complex

(A) Wild-type HEK 293 cells or HEK 293 cells transiently expressing *myc*–GRIP1 were lysed, and an immunoprecipitation with anti-*myc* antibody and an in vitro kinase assay were performed. In samples from *myc*–GRIP1-expressing cells, a phosphorylated band is observed that exactly matches the position of GRIP1. Exogenously added, bacterially expressed GST–GRIP1 is phosphorylated by the GRIP1-associated kinase activity present only in GRIP1 precipitates. In contrast, the cytoplasmic region of ephrinB1, added as GST–ephrinB1cyto, is not utilized as substrate by the GRIP-associated kinase activity.

(B) Phosphoamino acid analysis of in vitro phosphorylated GRIP1. Thin layer chromatography on hydrolyzed phosphorylated GRIP1 shows predominant phosphorylation on serine residues.

(C) Wild-type HEK 293 cells or HEK 293 cells transiently expressing *myc*–GRIP1 were lysed, and increasing amounts of the ephrinB1 peptide pepC7 (see legend to Figure 4A) were added to the lysates. GRIP was immunoprecipitated with anti-*myc* antibodies and subjected to an in vitro kinase reaction. No reduction of GRIP1 phosphorylation was observed in the presence of pep C7, suggesting no impairment of or competition with the associated kinase activity.

(D) HEK 293 cells transiently expressing *myc*–GRIP1 either alone or in the presence of HA–ephrinB1wt were lysed, and EphB2–Fc receptor bodies were used to precipitate HA–ephrinB1wt from the lysates followed by an in vitro kinase reaction. Coprecipitated and phosphorylated GRIP1 is observed only in the precipitate from HA–ephrinB1wt-containing cell lysate, due to isolation of a complex containing EphB2–Fc, ephrinB1wt, GRIP1, and the associated kinase activity. Panels below show the presence of ephrinB1-coprecipitated GRIP1 and EphB2-precipitated ephrinB1 as well as the levels of transfected GRIP1 in the total cell lysates.

of ephrinB1 and GRIP2 required the cytoplasmic domain of ephrinB1.

Under physiological conditions, ephrinB1 ligands bind and are activated by EphB receptors to trigger reverse ephrin phosphorylation. We therefore asked if EphB receptor contact could induce coalescence of the small raft domains at the plasma membrane and would lead to copatching of GRIP proteins. We expressed wild-type ephrinB1 together with GRIP2 in BHK cells and stimulated the cells with soluble EphB2–Fc "receptor bodies." Immunofluorescence using antibodies directed against the fused Fc portion revealed the redistribution of EphB2–ephrinB1 complexes into patches (Figure 6). Again, GRIP2 was recruited by ephrinB1 into patches that essentially overlapped with the EphB2–Fc signal (Figure 6). Binding of EphB2–Fc to cells expressing ephrinB1ΔC caused the formation of patches but no redistribution and recruitment of GRIP2. These results showed that EphB2 receptor binding to the extracellular domain of ephrinB1 caused the redistribution of ephrinB1–GRIP2 complexes into large, microscopically visible patches on the cell surface. Hence, under conditions in which ephrinB ligands are thought to trigger reverse signaling into the ligand-expressing cell (Holland et al., 1996; Brückner et al., 1997), patching of ephrinB molecules in the membrane can be observed microscopically. GRIP proteins are tightly associated with the cytoplasmic domain of ephrinB ligands, suggesting a role for GRIPs in recruiting or stabilizing signaling molecules.

GRIP Proteins Recruit a Serine/Threonine Protein Kinase to the Ephrin–GRIP Complex

To gain further support for a signaling role of GRIP, we investigated whether GRIP1 was associated with a protein kinase that may be capable of phosphorylating serine residues primarily located in the linker regions. We immunoprecipitated *myc*–GRIP1 from transiently transfected HEK 293 cells using anti-*myc* antibodies and performed an in vitro kinase assay on the IPs (Figure 7). Samples derived from *myc*–GRIP1-expressing, but not from untransfected, HEK 293 cells displayed a phosphorylated protein that exactly comigrated with GRIP1, suggesting that GRIP1 became phosphorylated by a coprecipitated, endogenous protein kinase activity. To verify that this kinase was specifically associated with GRIP1, we added a bacterially expressed glutathione S-transferase– (GST–) GRIP1 fusion protein to the kinase reaction. Indeed, GST–GRIP1 served as a substrate for phosphorylation only in GRIP1-containing but not in negative control IPs, indicating that the presence of the kinase depended on the presence of GRIP1. In contrast, the cytoplasmic region of ephrinB1, when added as GST fusion protein, was not used as a substrate by a GRIP-associated kinase activity. Phosphoamino acid analysis of in vitro phosphorylated GRIP1 revealed that GRIP1 was predominantly phosphorylated on serine residues, indicating that the coprecipitating kinase had serine/threonine specificity (Figure 7B).

We next investigated whether this GRIP-associated kinase could be recruited into an ephrinB–GRIP complex. First, we examined if association of the protein kinase activity was stable in the presence of a peptide corresponding to the C-terminal seven residues of ephrinB1. As depicted in Figure 7C, no reduction in GRIP1 phosphorylation was observed when lysates were incubated with increasing amounts of ephrinB1 peptide, suggesting that it did not interfere or compete with the associated kinase activity. Previous experiments had shown that this peptide potently inhibits binding between ephrinB1 and GRIP1 (Figure 3). We

next used HEK 293 cells cotransfected with ephrinB1 and GRIP1 to assay for the presence of the GRIP-associated kinase in an ephrinB1–GRIP1 complex. Soluble EphB2–Fc receptor bodies were used to precipitate ephrinB1 from cell lysates, and an in vitro kinase assay was performed to check for phosphorylation of GRIP1, serving as an indicator of complex formation between ephrinB1, GRIP1, and the GRIP-associated kinase. Indeed, GRIP1 coprecipitation and phosphorylation was observed only in the presence, but not in the absence, of ephrinB1, indicating the formation of a tetrameric complex between an EphB receptor ectodomain, its cognate ephrinB1 ligand, the PDZ adaptor GRIP1, and an associated protein kinase (Figure 7D). Our findings suggest that GRIP binding to ephrinB1 in the plasma membrane recruits a serine/threonine protein kinase, thereby organizing a signaling complex in raft lipid microdomains.

Discussion

The GRIP Family of PDZ Adaptor Proteins, Intracellular Targets of EphrinB Ligands

We identified GRIP (here referred to as GRIP1) and a highly related protein, which we have termed GRIP2, as intracellular targets of all ephrinB ligands. GRIP2 is the full-length version of AMPA receptor–binding protein (ABP), which was recently shown to bind AMPA receptors at synapses and to heteromultimerize with GRIP1 (Srivastava et al., 1998). Our isolation of the full-length cDNA of GRIP2 has revealed its close similarity to GRIP1 and would suggest considering ABP/GRIP2 as the second member of the GRIP family of proteins. We have demonstrated that both GRIP members play a role as targets of ephrinB proteins. Torres et al. (1998) have recently shown that other PDZ proteins, like the protein kinase Cα-interacting Pick1 and the Syndecan-interacting protein Syntenin, can interact with ephrinB ligands, demonstrating that PDZ proteins colocalize with and possibly regulate the clustering of ephrins and their Eph receptors at synaptic sites. Here, we suggest a signaling function for GRIP proteins downstream of ephrinB ligands in the novel context of ephrinB raft microdomain localization. Expression data from rat embryos suggest that ephrin–GRIP interactions may already have importance during patterning processes in the developing embryo.

EphrinB Reverse Signaling and Raft Microdomains

In support of the idea that ephrinB ligands engage in reverse signaling, we found that ephrinB1 is confined to lipid microdomains, so-called rafts. Raft association was observed in heterologous HEK 293 cells and, more importantly, in mouse brain homogenates. Rafts are enriched in signaling molecules such as Src family tyrosine kinases, Ras, and trimeric G proteins and are thought to act as platforms for signaling events (Simons and Ikonen, 1997). Moreover, binding of the physiological receptor EphB2 to the ephrinB1 extracellular domain causes the clustering of ephrinB1 into larger patches of raft domains on living cells. Clustering into raft domains was hypothesized to locally concentrate raft-associated proteins and to trigger signal transduction (Harder et al., 1998). Recent examples provided functional evidence that this is indeed the case. In resting T lymphocytes, the T cell receptor (TCR) is not associated with detergent-resistant fractions, while the CD4 receptor and Src kinases are found in rafts. Upon TCR activation, TCR moves into the Src kinase–rich raft domain, where it becomes tyrosine phosphorylated, which is an essential early step in T cell activation (Montixi et al., 1998; Xavier et al., 1998). Antigen-mediated cross-linking of high-affinity IgE receptors causes the redistribution from a uniform to a punctate plasma membrane distribution in living cells (Holowka and Baird, 1996; Stauffer and Meyer, 1997). IgE receptor effector molecules such as the tyrosine kinase Syk and phospholipase Cγ1 colocalize in these distinct membrane microdomains, indicating that IgE receptor–mediated signal transduction is spatially restricted and requires raft patches (Stauffer and Meyer, 1997).

Stimulation of ephrinB ligands with their physiological EphB2 receptor triggers tyrosine phosphorylation of the ephrinB cytoplasmic domain by an unknown tyrosine kinase (Holland et al., 1996; Brückner et al., 1997). Further studies will show if this kinase is associated with raft microdomains and if the fusion of raft domains driven by receptor-mediated clustering of ephrinB molecules represents the mechanism for the recruitment and activation of this kinase, resulting in ephrinB tyrosine phosphorylation.

GRIP Proteins and Ephrin Signaling

GRIP proteins colocalize with ephrinB1 raft patches, independent of whether the ephrinB patches were generated by antibody cross-linking or EphB2 receptor binding. In unstimulated cells, microscopically visible coclusters of ephrinB1 and GRIP proteins were very rarely observed, indicating that this process is regulated under physiological conditions. Moreover, copatching required the cytoplasmic domain of ephrinB1, ruling out the possibility that GRIP unspecifically aggregates in these patches. We did, however, observe intracellular aggregates of GRIP proteins in cells expressing high levels of GRIP alone, indicating that GRIP proteins may be involved in other protein–protein interactions. GRIP has already been proposed to act as a multi-PDZ scaffold protein similar to fly InaD, which assembles a signaling complex for photo transduction comprising eye protein kinase C (eyePKC), its potential substrate phospholipase Cβ, and ion channels (Ranganathan and Ross, 1997; Tsunoda et al., 1997). Here, we demonstrate that GRIP is associated with a serine/threonine kinase activity, which, in addition, is able to utilize GRIP as an in vitro substrate. Moreover, this protein kinase is part of an ephrin–GRIP complex, supporting the view that GRIPs serve as scaffold or docking molecules. Together with InaD and the PKC-binding protein Pick1 (Staudinger et al., 1995, 1997), the GRIP family proteins now provide another example of PDZ domain proteins localizing protein kinases. Since ephrinB molecules are thought to act as receptors in mediating reverse signaling, the recruitment of signaling molecules by GRIP proteins to the sites of ephrinB ligands could link ephrinB signaling to cellular kinase cascades, propagating signal transduction or remodeling cytoskeletal organization.

Ephrin Clustering and Forward Signaling

An important variable in ephrin ligand function is the postulated multimerization of ephrin molecules in cellular membranes (Davis et al., 1994). Depending on the state of ephrin oligomerization, differential Eph receptor signaling complexes and biological effects were shown to be induced (Stein et al., 1998). Could GRIP regulate ephrinB multimerization or clustering? GRIP has been shown to be localized in clusters with AMPA receptors at excitatory synapses in primary neurons (Dong et al., 1997). However, more recently, AMPA receptors were shown to have diffuse synaptic localization, and GRIP2/ABP was reported to interact with AMPA receptors without inducing apparent clustering (Allison et al., 1998; Srivastava et al., 1998). It is therefore possible that GRIP proteins mainly function to target AMPA receptors to synapses rather than to cluster them. Our own work has not provided evidence for the involvement of GRIP proteins in clustering ephrinB ligands. However, our cell system may lack an essential third component that would allow GRIP proteins to aggregate ephrinB molecules. Alternatively, ephrinB oligomers induced by GRIP may be too small to be detected yet be sufficient to trigger EphB receptor activation in a neighboring cell.

An attractive model would be that multimerization of ephrinB ligands is controlled by the aggregation state of GRIP proteins. Aggregation of PDZ domain proteins may be mediated by homo- or heteromultimerization of PDZ domain proteins or by interaction with cytoskeletal components (Kim, 1997). Consistent with this hypothesis, we observed some homophilic binding of GRIP1 in the yeast two-hybrid assay (data not shown), and heteromultimers of GRIP1 with GRIP2/ABP were reported (Srivastava et al., 1998). Therefore, additional interactions of GRIP proteins could control its aggregation state and the potential of ephrinB ligands to trigger "forward" signaling through Eph receptors.

The Role of Phosphorylation

In at least one case, PDZ interactions with C-terminal target sites have been shown to be influenced by phosphorylation. Phosphorylation by protein kinase A of a serine residue at position −2 of the C-terminal PDZ target site of the inward rectifier potassium channel Kir2.3 abolishes binding of the PDZ domain protein PSD-95 (Cohen et al., 1996). In ephrinB ligands, C-terminal positions −2 and −3 are occupied with two tyrosine residues. EphrinB molecules are known to be inducibly tyrosine phosphorylated within their cytoplasmic domains when engaged in reverse signaling or intracellular cross-talk (Holland et al., 1996; Brückner et al., 1997). In contrast, binding of GRIP proteins already occurs in the absence of tyrosine phosphorylation and Eph receptor contact. Whether phosphorylation of the C-terminal tyrosine residues changes the binding affinity to GRIP proteins in positive or negative ways is under current investigation. Our initial observation that GRIP proteins are partially removed from cellular membranes by treating cells with the phorbol ester TPA, a well-known activator of PKC (Kikkawa et al., 1983), suggests that ephrinB–GRIP interactions are modulated by intracellular signaling activities controlling ephrin and/or GRIP proteins that could involve phosphorylation events or changes in second messengers.

EphrinB ligands may activate multiple distinct signaling pathways—those that depend on tyrosine phosphorylation and are mediated by phosphotyrosine-binding proteins and others that are independent of tyrosine phosphorylation and are mediated by PDZ domain proteins. Alternatively, ephrinB signaling initiated by tyrosine phosphorylation could require a GRIP-controlled protein complex to relay downstream signaling events.

Ephrin–GRIP Interactions during Embryogenesis

During embryonic development, GRIP1 is expressed early on, while GRIP2 expression is somewhat delayed, with high levels appearing at later stages of embryogenesis. The availability of different GRIP members could regulate the composition of ephrin–GRIP complexes. In addition, other PDZ domain proteins may compete for ephrin binding, or proteins with similar target sites may compete for GRIP binding. This could explain why ephrin–GRIP complexes apparently cannot be detected at very high stoichiometry in the mouse embryo. Considering that both GRIP members are more divergent in domains other than the ephrin-binding PDZ6, it is conceivable that the nature of the ephrin–GRIP complex could regulate ephrin signaling and/or ephrin oligomerization.

Genetic evidence from EphB2 loss-of-function experiments suggested a role for reverse ephrinB signaling in the developing mouse embryo during anterior commissure formation. In this structure, ephrinB ligands are expressed by cortical neurons that form the anterior commissure and are repelled by ventral forebrain cells that express the corresponding EphB2 and EphB3 receptors (Henkemeyer et al., 1996). Our comparative in situ hybridization analysis revealed colocalization of ephrinB1 and ephrinB2 with both GRIP1 and GRIP2 in specific layers of the embryonic neocortex. These observations are consistent with GRIP proteins being involved in ephrinB signaling during anterior commissure formation.

Recently, ephrinB2 was shown to be required for the remodeling of the vascular network during early embryogenesis (Wang et al., 1998; Adams et al., 1999). Thus, the colocalization of ephrinB2 (and to a lesser extent, of ephrinB1) with GRIP1 on dorsal aorta and aortic branches is intriguing and suggests a role for ephrin–GRIP interactions in blood vessel formation.

We have demonstrated the association of ephrinB ligands with raft microdomains and their clustering into raft patches upon EphB2 receptor stimulation. Therefore, we would like to propose a mechanistic model for EphB receptor–induced ephrinB tyrosine phosphorylation being mediated by raft stabilization and subsequent kinase activation. As GPI-anchored proteins are localized in raft microdomains (Brown and Rose, 1992), and their patching can lead to kinase activation (Brown, 1993; van den Berg et al., 1995), we speculate that membrane clustering of GPI-anchored ephrinA ligands may result in an analogous reverse activation of tyrosine kinases. Furthermore, we have shown that GRIP proteins are concentrated in ephrinB-containing lipid domains, recruiting at least a serine/threonine protein kinase into the ephrinB–GRIP complex. This suggests a role for GRIP family proteins as scaffolds for the assembly of

protein complexes downstream of ephrinB molecules with potential implications in signal transduction or cytoskeletal organization. Although it is expected that ephrin–GRIP interactions will have important roles in ephrin reverse signaling, they may also, e.g., via oligomerization, affect forward signaling by activated Eph receptors.

Experimental Procedures

Isolation of GRIP1 by the Yeast Two-Hybrid Screen

The 82 C-terminal amino acids of the cytoplasmic region of human ephrinB1 (NH2-RKRHRK...NIYYKV-COOH [single letter code]) were amplified by PCR and cloned into pAS2 (pAS1-CYH2) (from S. Elledge) with NdeI/BamHI to create the bait construct pKB3. We screened 3×10^6 transformants of a human fetal brain cDNA library (Clontech) as described (Hoffman and Winston, 1987; Gietz et al., 1992) with Y190 (from S. Elledge). Positive clones were detected by β-galactosidase activity in the in vivo plate assay: yeast colonies were grown on nitrocellulose membranes and shifted to X-gal plates (containing 65 μg/ml X-gal, 100 mM Na phosphate [pH 7.0]) with blue color developing after 6–36 hr. Library plasmids were tested for interaction with unrelated baits, including pAS1-Cdk2 (S. Elledge), pAS1-SNF1 (S. Elledge), and pAS1-lamin (S. Fields). To check for interaction with the C terminus of ephrinB1, we constructed a bait containing the cytoplasmic region of human ephrinB1 lacking the last seven amino acids (pJP95; NH2-MLRKR...EMPPQSP-COOH) and a bait containing the cytoplasmic region of human ephrinB1 in which the last seven amino acids of ephrinB1 were exchanged with the sequence SSIESDV, corresponding to the C terminus of the NMDA receptor subtype R2A (pJP96). The library plasmid containing a partial human GRIP1 cDNA clone was sequenced, and the sequence of the open reading frame was submitted to GenBank.

Cloning of *GRIP2* cDNA Clones

The following degenerate primers corresponding to the published rat GRIP sequence were used to amplify a GRIP-related PCR fragment from rat brain cDNA: GRIP4, AGAGAATTCGAGAT(ATC)GA(AG)TTTGA(TC)GT(GATC)GC, and GRIP11rev, AGATCTAGAACAA(GA)(GA)CA(GA)CA(GA)TC(GA)AA(GA)TC. The resulting PCR fragment was used to screen a P15 rat hippocampal cDNA library. Those clones corresponding to the novel GRIP2 cDNA were sequenced, and the sequence was submitted to GenBank.

GRIP1 and GRIP2 Deletion Analysis

GRIP deletion analysis was performed in yeast strain SFY526 (Bartel et al., 1993). GRIP1 prey deletion constructs for mapping the interaction sites with ephrinB1 were generated by introducing artificial sites at the 5′ and 3′ ends of PCR-amplified regions of GRIP1. All of them were cloned into pGADGH (Clontech). The sequences at the N and C termini and their amino acid positions, corresponding to the published rat sequence (Dong et al., 1997), are as follows: G1/3p-5 (pJP107), (268) TTSMCC...SGAIIY (671); G1/4–6 (pJP121), (430) TSPRGT...KLSDVY (793); G1/6–7 (pJP113), (667) GAIIYT...EPTNTL (1112); G1/6p-7 (pJP109), (712) AIHIGDR...EPTNTL (1112); G1/5+6 (pJP128), (560) VAESVI...KQTDAQ (757); G1/6 (pJP127), (657) EDNSDE...KQTDAQ (757); G1/5p-6p (pJP122), (595) GDPLVI...GAIHIG (717); and G1/6p (pJP123), (657) EDNSDE...GAIHIG (717). GRIP2 deletion constructs were as follows, with amino acid positions as shown in Figure 1: G2/1–3 (including insert in linker), (1) MLA VSL...MSPRTT (422); G2/4–6, (418) MSPRTT...HFSPAV (779); G2/7, (902) VQSVAV...SSPQMI (1043); G2/5+6, (542) EIEFDV...KQLDRP (743); G2/6, (643) EDNSDE...KQLDRP (743); and G2/6+7, (643) EDNSDE...SSPQMI (1043). All prey deletion constructs were verified by sequencing.

In Situ Hybridization Analysis

In situ hybridization with ^{35}S-labeled antisense oligonucleotides was performed as described (Wisden and Morris, 1994). In short, oligonucleotides were 3′labeled by the addition of ^{35}S-labeled dAMP residues by terminal transferase (Boehringer Mannheim). Hybridization was in 50% formamide in 4× standard saline citrate (SSC), 10% dextrane sulfate at 42°C with probe used at 1 pg/μl. Sections were washed in 1× SSC at 60°C. Exposure time was 18 days on Kodak Biomax XR film. Hybridization with a 100-fold excess of unlabeled oligonucleotide in addition to the corresponding labeled probe was used to define nonspecific hybridizations.

The oligonucleotides used were:

ephrinB1, TGGTAGTCAACTGCTCAGGCGTTACGGCATTTGGAT;
ephrinB2, TACCAGGCCTTGTCCGGGTAGAAATTTGGAGTTCGA;
ephrinB3, ATGGGCATTTCAGACACAGGTTTTCGGGGGACAGC;
GRIP1, GGGCAGAGTGTTGCTGCGAGTCGTTTGGCTATAGTG; and
GRIP2, ATGATGGGGTCAAAAGGTTCCTCTGTACCAGAGATGGTG.

Cell Culture and Expression Constructs

HEK 293 cells were cultured and transfected by the calcium phosphate transfection method according to standard procedures. Plasmids were used at 5 μg/10 cm culture dish except for *myc*–GRIP1 (2.5 μg/dish). Cells were used 36–48 hr after transfection. To generate HA–ephrinB1 (pJP104), a hemagglutinin tag was introduced after the ephrinB1 leader peptide. The sequence in the junction is as follows: WALCRLAYPYDVPDYASLTPLAKNL (ephrinB1 sequence underlined). An artificial BamHI site was introduced in the sequence at the 5′ end of the ephrinB1 coding sequence and at one EcoRI site after the stop codon in order to clone it into pcDNA3 (Invitrogen). HA–ephrinB1ΔC (pJP105) was constructed in the same way, but the sequence of the truncated C terminus was as follows: KHTQQRAAA. Since constructs for wild-type ephrinB3 were not expressed well in HEK 293 cells, chimeric proteins between ephrinB1 and ephrinB3 were generated following protocols described previously (Labrador et al., 1997). HA–ephrinB3 (pJP125) was generated by fusing the extracellular domain of ephrinB1 to the transmembrane and cytoplasmic regions of ephrinB3. The sequence at the junction is as follows: FFNSKV/AVAGAA (ephrinB1 sequence underlined). HA–ephrinB3ΔC (pJP126) was generated by fusing the extracellular domain of ephrinB1 to the transmembrane region of ephrinB3 (sequence at the C terminus, ESRHPGP). To generate GFP–ephrinB1 (pJP136) or GFP–ephrinB1ΔC (pJP139), a similar procedure as for HA–ephrinB1 was followed, and pJP104 (HA–ephrinB1) or pJP105 (HA–ephrinB1ΔC) were used as templates to include the HA tag right after the GFP. The sequence at the junction between GFP and ephrinB1 is as follows: **GMDELYK**LAYPYDVPDYASLTPLAKNL (GFP sequence in bold, and ephrinB1 sequence underlined).

To generate pKB23 for *myc*–GRIP1, GRIP1 cDNA was isolated from the pGAD10 library plasmid by partial EcoRI digest and cloned into pcDNA3 with an N-terminal double *myc* tag. For *myc*–GRIP2 (pKB33), using PCR, an artificial EcoRI site was created right before the ATG to clone it into the same vector with an N-terminal double myc-tag.

Coimmunoprecipitations and Western Blots

For coimmunoprecipitation of ephrins and GRIPs, cells were washed twice with phosphate-buffered saline (PBS) and lysed (50 mM Tris [pH 7.5], 140 mM NaCl, 10 mM NaF, and 0.5% Triton X-100). For immunoprecipitation of GRIP1, cells were lysed in 50 mM Tris (pH 7.5), 50 mM NaCl, 50 mM NaF, and 1% Triton X-100. Protease inhibitors and vanadate were added as described (Brückner et al., 1997). For immunoprecipitations and Western blots, the following antibodies were used: mouse monoclonal anti-HA (12CA5), mouse monoclonal anti-*myc* (9E10), rabbit polyclonal anti-ephrinB1 A,B (previously described as anti-Lerk2 A,B [Brückner et al., 1997]), anti-GRIP1 (5118) raised against GST–GRIP1, rabbit polyclonal anti-caveolin (anti-cav) (N20) (Santa Cruz), and mouse monoclonal and anti-transferrin receptor (anti-TfR) H68.4 (Zymed). EphB2–Fc (previously described as Cek5–Fc [Brückner et al., 1997]) was utilized as a precipitation tool. Precipitations were performed with protein A- or protein G-Sepharose (Pharmacia). Immunoprecipitations were incubated 2–3 hr at 4°C and washed four times with lysis buffer. In some samples, ephrinB C-terminal peptide C7 (CANIYYKV*) was added to the lysate at 0.2 and 20 μg/ml. SDS–PAGE and immunoblots were done according to standard procedures with Protogel (National Diagnostics) and secondary antibody–HRP conjugates (Amersham, Dianova). Blots were developed by enhanced chemiluminescence (Amersham).

Membrane Fractionation and Triton X-100 Flotation Experiments

Transfected HEK 293 cells on 3 cm culture dishes were washed and scraped in PBS and after pelleting, resuspended in 200 μl of TNE buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 5 mM EDTA) containing protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin, at 25 μg/ml each). Cells were homogenized at 4°C by passing five times through a 25G needle and ten times through a 27G needle. In a TLS55 centrifuge tube (Beckman, Palo Alto, CA) the extract was adjusted to 30% Optiprep (Nycomed Pharma, Oslo, Norway) and overlayered with 1.6 ml of 25% Optiprep (in TNE) and 200 μl of TNE. After centrifugation (1 hr, 50,000 rpm, 4°C), five fractions were collected from the top, with the first one representing the membrane fraction (0%/25% interface), and the bottom fraction containing soluble proteins (the three middle fractions did not contain significant amounts of protein; data not shown). After incubation with 1% Triton X-100 at room temperature, proteins were precipitated with tricarboxylic acid (TCA) and analyzed by SDS–PAGE and Western blotting. Stimulations with TPA were performed at 1 mM TPA overnight (12–14 hr) prior to homogenization.

For the analysis of detergent-insoluble complexes in flotation gradients, HEK 293 cells on 3 cm culture dishes were scraped and pelleted as described above and cooled on wet ice. Then cells were extracted with 150 μl of precooled TXNE (TNE containing 0.1% Triton X-100 and protease inhibitors) for 20 min on ice. Subsequently, the extract was brought to 35% Optiprep in a SW60 centrifuge tube (Beckman) by adding 210 μl of 60% Optiprep/0.1% Triton X-100 and overlayered with 3.5 ml 30% Optiprep in TXNE and 300 μl TXNE. After centrifugation (4 hr, 40,000 rpm, 4°C), six fractions were collected from the top, TCA precipitated, and analyzed by PAGE and Western blotting.

To analyze detergent-insoluble complexes from adult brain, a membrane fraction was prepared as follows. Two brains (0.83 g tissue) were cut in small pieces and homogenized in a Dounce with 3 ml of ice-cold homogenization buffer (HB; 20 mM Tris-HCl [pH7.4], 50 mM NaCl, 250 mM sucrose, 1 mM DTT, and 0.5 mM vanadate; chymostatin leupeptin anti-pain pepstatin). The extract was then passed three times through a 22G needle. Large aggregates were pelleted for 10 min in an Eppendorf centrifuge (3,000 rpm, 4°C). The pellet was reextracted with 1 ml HB by passing through a 22G needle and spun as above. The supernatants were pooled and adjusted to 35% Optiprep (Nycomed Pharma), split into two SW40 centrifuge tubes, and overlayered with 2.5 ml of 30%, 20%, and 5% Optiprep, all in HB containing only 0.1 mM vanadate. After centrifugation for 3 hr (40,000 rpm, 4°C; SW40 rotor, Beckman), 12 fractions were collected from the top. One-seventh (300 μl) of the light membrane fraction recovered from the 5%/20% interface was used per detergent extraction. Membranes were extracted by addition of 200 μl HB containing 0.25% of Triton X-100 on ice, adjusted to 35% Optiprep, and overlayered with 2.5 ml 30% Optiprep and 500 μl HB, all containing 0.1% Triton X-100. Gradients were centrifuged and analyzed as above.

Cholesterol depletions with 10 mM cyclodextrin were performed as described (Scheiffele et al., 1997). When 0.5% Triton X-100 instead of 0.1% Triton X-100 was used for extraction, similar results were obtained for the flotation of EphrinB1; however, the interaction with GRIP2 was reduced (data not shown).

Immunofluorescence and Patching Experiments

For immunofluorescence, transfected BHK cells (strain CCL10, American Type Culture Collection) were fixed for 5 min with 3.7% formaldehyde in PBS at 7°C followed by incubation in methanol at −20°C for 5 min. Unspecific binding sites were blocked by incubation with 2 mg/ml bovine serum albumin (BSA) in PBS (PBS–BSA) for 20 min. Primary and secondary antibodies (conjugated to FITC or rhodamine, Dianova) were diluted in PBS–BSA and incubated with the blocked cells for 30 min followed by three washes with PBS–BSA, and cells were mounted in 20 mM Tris-HCl (pH 8.0), 80% glycerol, and 4% N-propyl gallate.

For patching, either affinity-purified anti-GFP antibodies or recombinant EphB2–Fc (Brückner et al., 1997) were used. Cells were washed with PBS and incubated for 30 min with rabbit anti-GFP (0.75 μg/ml in minimal essential medium [MEM] containing 0.35 g/l carbonate, 2 mg/ml BSA, and 20 mM HEPES [MEM–BSA]) followed by an FITC-conjugated goat anti-rabbit antibody (15 μg/ml in MEM–BSA) for 30 min at 12°C. Alternatively, cells were incubated with EphB2–Fc (4 μg/ml in MEM–BSA) at 12°C. Under these conditions, no significant internalization of the patched proteins occurred. Subsequently, cells were fixed as described above, and GRIP2 was detected by immunofluorescence staining with the anti-*myc* monoclonal 9E10 (7.5 μg/ml in PBS–BSA) and a rhodamine-conjugated goat anti-mouse antibody (15 μg/ml in PBS–BSA). EphB2–Fc was detected with FITC-conjugated goat anti-human Fc antibodies (15 μg/ml in PBS–BSA). When GFP fusion proteins on the cell surface were clustered by using FITC-conjugated secondary antibodies, the GFP fluorescence in the fixed cells was quenched by incubation with 50 mM NH_4Cl in PBS.

Pictures were taken with a confocal microscope (Leica DM DRB). The laser power for each channel was set such that no significant bleed through into the other channel was observed. In each picture, eight sections from the top to the bottom of the cell were taken and projected onto each other. Images were processed with Photoshop software (Adobe Systems, CA). For better visualization, images showing the single color channels are displayed in gray scale.

Kinase Assays and Phosphoamino Acid Analysis

For in vitro kinase assays, IPs were washed with kinase buffer (20 mM HEPES [pH 7.2], 5 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT). Kinase reactions were performed in the presence of 10 μCi [^{32}P]γATP for 8 min at 30°C. In some samples, purified GST–GRIP1 (200 ng) or GST–ephrinB1cyto (1 μg) was added as an exogenous substrate. Samples were resolved by SDS–PAGE and directly exposed or transferred to nitrocellulose and exposed before probing with antibodies. For phosphoamino acid analysis, samples were transferred to a polyvinylidene difluoride membrane (Immobilon), and the respective band was cut out. Phosphoamino acids were analyzed by HCl hydrolysis of the protein followed by thin layer chromatography according to standard procedures (Kamps, 1991).

GST–ephrinB1cyto was described previously (GST–Lerk2cyto) (Brückner et al., 1997). For GST–GRIP1 (pKB34), GRIP1 cDNA was isolated from the pGAD10 library plasmid by partial EcoRI digest and cloned into pGEX4T1. GST–GRIP1 was purified from bacteria with N-laurylsarcosine (Frangioni and Neel, 1993).

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GenBank Accession Number

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